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Graphene oxide based ultrasensitive flow-through chemiluminescent immunoassay for sub-picogram level detection of chicken interferon-γ

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ABSTRACT

The quantitative detection of chicken interferon-γ (ChIFN-γ) released by T cells after in vitro stimulation is a good evaluation of cell-mediated immunity in chickens after infection or vaccination. This work reports a new flow-through the chemiluminescent (CL) immunoassay method for rapid and specific determination of ChIFN-γ at a level of sub-picogram per milliliter. The biocompatible graphene oxide nanosheet is introduced into CL immunoassay for highly efficient immobilization of capture antibody. The detection limit of the proposed method at a signal to noise ratio of 3 is 0.36 pg ml^{-1}, which is 138-fold lower than the current lowest value of 50 pg ml^{-1} for ChIFN-γ. Coupling with a flow-through system, the whole immunoassay process can be completed within 25 min. The resulting ChIFN-γ immunosensor shows excellent detection and fabrication reproducibility, good specificity and stability. The assay results of nature ChIFN-γ samples with the proposed method are in an acceptable agreement with the reference values. Compared to the present assay methods, this method is more flexible, simple, rapid and sensitive. The aim of this work is to demonstrate that the ultrasensitive, specific and rapid CL immunoassay format can become a very potential application for quantifying ChIFN-γ and further studying its role in immune response in poultry.

1. Introduction

Interferon-γ (IFN-γ), a cytokine produced primarily by activated T lymphocytes and natural killer cells in response to mitogen or antigen stimulation, plays a critical role in resistance against various mammalian and avian pathogens (Farrar and Schreiber, 1993; Billiau, 1996; Schultz and Chisari, 1999; Liu et al., 2010a). Like its mammalian counterpart, chicken IFN-γ (ChIFN-γ, a molecular weight of 17–20 kD) is sensitive to heat and acid pH treatment (Digby and Lowenthal, 1995). ChIFN-γ also enables to activate macrophages, enhance the expression of MHC (major histocompatibility complex) class-II antigen and neutralize viral replication (Digby and Lowenthal, 1995; Song et al., 1997). Previous studies have shown that the measurement of ChIFN-γ released by T cells after in vitro stimulation is a good evaluation of cell-mediated immunity (CMI) in commercial chickens after infection or vaccination (Martin et al., 1994; Karaca et al., 1996; Breed et al., 1999). The biological assays of ChIFN-γ are mainly based on the measurement of nitric oxide produced by the activation of the HD11 macrophage cell line (Sekellick et al., 1994; Digby and Lowenthal, 1995; Karaca et al., 1996), which are laborious, time-consuming, difficult to standardize, and sensitive to buffers or other cytokines present in the supernatants (Lambrecht et al., 2000). Although the sensitive and specific microplate enzyme-linked immunosorbent assay (ELISA) methods for ChIFN-γ have been developed (Lambrecht et al., 2000, 2004; Yun et al., 2000), the assay formats still suffer from some limitations such as labor-intensive and time-consuming manipulations and inadequate detection sensitivity. In addition, only a few researches of ELISA have been reported for highly sensitive determination of ChIFN-γ over the past decade. Thus it is very urgent to develop an ultrasensitive, specific, rapid and reliable assay method for quantitative detection of ChIFN-γ.

Chemiluminescent (CL) immunoassay, in particular enzyme labeled CL immunoassay, has been extensively exploited in recent years due to its high sensitivity, wide dynamic range and suitability for miniaturization (Fall et al., 2003; Zhou et al., 2006; Wolter et al., 2008; Wu et al., 2009). The basic principle of CL enzyme immunoassay is that the CL intensity produced by the enzyme catalytic chemical reaction is directly proportionate to the amount of analytes present in a sample (Liu et al., 2010b). Due to its small sample consumption, simplified handling step, acceptable reusability, good reproducibility, and easy automation for high sample throughout

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(Gunaratna and Wilson, 1993; Nandakumar et al., 2000), flow-injection immunoassay has attracted increasing attention in environmental monitoring (Jain et al., 2004), food safety (Delehanty and Ligler, 2002; Knecht et al., 2004), pharmaceutical analysis (Eremenko et al., 1998; Yang et al., 2002), and clinical diagnosis (Fu et al., 2006; Yang et al., 2008; Tang et al., 2009). Here, a CL immunoassay coupled with flow-through system was proposed for ultrasensitive, specific and rapid detection of ChIFN-γ by using graphene oxide as matrix for immobilization of capture antibody.

Graphene oxide (GO), a two-dimensional nano-carbon material produced by the oxidation of graphite, contains hydroxyl and epoxide functional groups on their basal planes, in addition to carboxyl groups located at the sheet edges (He et al., 1998). These functional groups make GO sheets strongly hydrophilic and thus allow them readily swell and disperse in aqueous media (Hirata et al., 2005). Due to its unique and excellent electronic, thermal, optical, mechanical properties and high surface area, GO has recently received tremendous attention in both experimental and theoretical scientific fields (Geim and Novoselov, 2007; Loh et al., 2010). As a novel nanomaterial that contains graphene-like sheets, GO provides a new avenue for fabricating excellent biosensors and biosensing devices (Loh et al., 2010; Guo et al., 2011; Bonanni et al., 2012; Morales-Narvaez and Merkoci, 2012). Biocompatible GO sheets as a sensing platform not only provide an abundant domain for biomolecules but also play a role of signal amplification detection (Du et al., 2010). In order to combine the advantages of GO with the good water permeability of chitosan (Kang et al., 2009), here, GO-chitosan composite was prepared to construct a highly efficient CL immunosensor for ultrasensitive and rapid flow-through CL immunoassay of ChIFN-γ. The composite provided a good biocompatibility and high capacity for loading of proteins. This designed flow-through CL immunoassay system showed low detection limit, wide linear range and rapid assay speed for high-throughput detection of ChIFN-γ. It could be successfully applied in the detection of practical ChIFN-γ sample without any pretreatment. This work provides a very promising and useful approach to rapidly quantify ChIFN-γ for good evaluation of CMI in commercial chickens.

2. Materials and methods

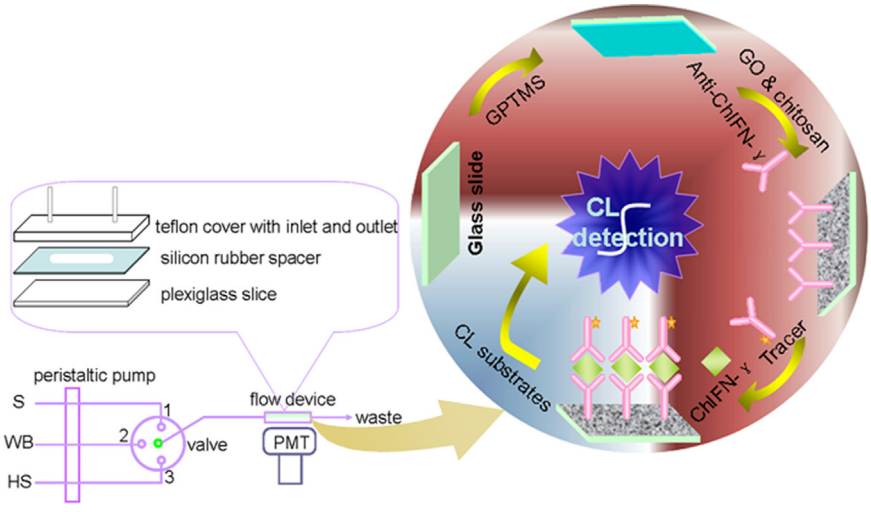
2.1. Materials and reagents

The purified recombinant ChIFN-γ protein from *Escherichia coli* (0.33 mg ml^{-1}), and purified monoclonal antibodies against ChIFN-γ named 3E5 (3.12 mg ml^{-1}) and 3E3 (3.24 mg ml^{-1}) were made by Jiangsu Key Lab of Zoonosis of Yangzhou University. Horseradish peroxidase (HRP), 3-glycidoxypropyltrimethoxysilane (GPTMS, 98%), chitosan and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Graphite powder, sodium borohydride ($NaBH_4$) and hydrogen peroxide (30%, H_2O_2) were bought from Sinopharm Chemical Reagent Co. Ltd. (China), and *p*-iodophenol (PIP) was from Alfa Aesar China Ltd. Luminol (Acros, Belgium) stock solution (0.01 M) was prepared in 100 ml of 0.1 M NaOH. Prior to use luminol and PIP stock solutions were mixed and diluted using 0.1 M pH 8.5 Tris–HCl buffer. The HRP substrate solution consisted of luminol (5 mM)-PIP (0.6 mM)-H_2O_2 (4 mM). Blocking buffer was 0.01 M pH 7.4 phosphate buffer solution (PBS) containing 1% BSA. To minimize unspecific adsorption, 0.05% Tween-20 was spiked into PBS as wash buffer (PBST).

2.2. Apparatus

The flow-through CL enzyme immunoassay system for ChIFN-γ was constructed and illustrated in Scheme 1. The Teflon tubes (0.8 mm i.d.) and silicon rubber tubes (1.0 mm i.d.) were used to connect all components in this system. All fluids were delivered with a multichannel bidirectional peristaltic pump. The introductions of different solutions into the flow system were performed using a multiposition valve with five inlets and one outlet. The flow device (Scheme 1) was composed of a Teflon cover (4.0 cm × 2.5 cm × 0.8 cm) with inlet and outlet, a silicon slice rubber spacer (2.0-mm thickness), and a transparent plexiglass slice (0.5-cm thickness), which was positioned in front of photomultiplier (PMT). The CL signals produced in flow device were measured with the PTM operated at −500 V. Instrument control and data record were performed using IFFM software package run under Windows 2003.

Flow-through CL measurements were performed with an IFFM-E Luminescent Analyzer (Remex Analytical Instrument Co. Ltd., Xi'an, China, http://www.chinaremex.com). Transmission electron micrographs (TEM) were obtained with a Hitachi S-4800 scanning electron microscope (Japan) at an acceleration voltage of 100 kV. Scanning electron micrographs (SEM) were obtained with a Hitachi S-4800 scanning electron microscope (Japan) at an acceleration voltage of 15 kV. The static water contact angles were measured with a contact angle meter (Rame-Hart-100) using droplets of the distilled water at 25 °C.



Scheme 1. Schematic illustration of the flow-through chemiluminescent immunosensing system for ChIFN-γ. (S) Sample, (WB) wash buffer, (HS) HRP substrate, (PMT) photomultiplier.

2.3. Synthesis of graphene oxide

GO was synthesized chemically according to the Hummers and Offeman method (Hummers and Offeman, 1958). Briefly, 5 g of graphite and 2.5 g of sodium nitrate were mixed into 15 ml of concentrated sulfuric acid. After the mixed solution was cooled to 0 °C in an ice-bath, 15 g of potassium permanganate was slowly added to the suspension. The addition rate was controlled carefully to prevent the temperature of the suspension from exceeding 20 °C. Then ice-bath was removed and the suspension was stirred for 30 min, where the temperature was kept at 35 °C. Subsequently, 230 ml of water was slowly stirred into the brownish-grey paste, causing an increase in temperature to 98 °C. The diluted suspension was maintained at this temperature for 15 min, then diluted with 700 ml of warm water and treated with 3% H_2O_2 to reduce the residual permanganate and manganese dioxide. The suspension was filtered while it was still warm to avoid precipitation of mellitic acid formed as a side reaction. The as-formed yellowish-brown filter product was washed carefully with warm water and then treated with resinous anion and cation exchanger to remove the remaining salt impurities. The dry form of GO was obtained by centrifugation and drying in vacuum at 40 °C.

2.4. Preparation of natural ChIFN-γ

Spleen of 8-week-old specific pathogen free chickens (provided by comparative medical center of Yangzhou University) was moved away sterilely, and single cell suspension was prepared according to the previous method (Lambrecht et al., 2004). Splenocytes were adjusted to 10^7 cells ml^{-1} in RPMI1640 (GIBCO) containing 10% (v/v) fetal bovine serum (Hyclone) and 5% (v/v) penicillin–streptomycin solution (Sino-American Biotechnology Company). 250 μl cells per well were transferred into flat-bottomed 24-well plates. Equal volumes of medium with 10% of inactivated fetal bovine serum containing Con A (24 μg ml^{-1}, Sigma) were added in triplicate. The cells were incubated at 4 °C in 5% CO_2, and cultures were incubated for 4 days. Negative controls received 250 μl RPMI1640 medium only. After 96 h of incubation, the supernatant were harvested for the measurement of ChIFN-γ production.

2.5. Preparation of HRP-labeled ChIFN-γ antibody

Coupling of the anti-ChIFN-γ antibody with HRP was performed according to the previously described method (Zhang et al., 2010). Firstly, 0.5 ml of 0.06 M fresh $NaIO_4$ solution was mixed with 0.5 ml of 5 mg ml^{-1} HRP solution and kept for 30 min at 4 °C, followed by adding 0.5 ml of 0.16 M glycol. Then, 1 ml of 3.24 mg ml^{-1} anti-ChIFN-γ antibody (3E3) was dispersed into the mixture, and the pH of the mixture was adjusted to 9.0 using 0.05 M pH 10.0 carbonate buffer saline. After reacting for 16–24 h at 4 °C, 0.2 ml of 5 mg ml^{-1} $NaBH_4$ solution was added and incubated for 2 h at 4 °C to produce HRP-labeled anti-ChIFN-γ, followed by dropwise addition of 0.2 ml of saturated ammonium sulfate to precipitate HRP-labeled anti-ChIFN-γ. After centrifugation at 4000 rpm for 15 min, the obtained bioconjugate was redispersed in 0.01 M pH 7.4 PBS and further purified by dialysis overnight to obtain 0.5 mg ml^{-1} solution of HRP-labeled anti-ChIFN-γ antibody.

2.6. Preparation of immunosensor for ChIFN-γ

A glass slide (2.5 cm × 0.4 cm × 0.1 cm) was silylanized with GPTMS to form active epoxy groups according to the previous method (Luzinov et al., 2000). 2.0 mg GO was dispersed in 1.0 ml 1.0 wt% chitosan solution with ultrasonication and then mixed with 200 μg ml^{-1} anti-ChIFN-γ (3E5). 30 μl of the resulting mixture was dropped on the silylanized slide to react at room temperature (RT) for 1 h and followed at 4 °C overnight. After washing three times with PBST, the left epoxy groups were blocked with blocking buffer for 12 h at 4 °C. The antibody immobilized slide was then fixed on the center area of the inner side of the Teflon cover in the flow device. The thickness of the film was less than 0.1 mm. The volume of the fabricated flow device was 90 μl (2.5 cm × 0.4 cm × 0.09 cm). The prepared ChIFN-γ immunosensor was stored in at 4 °C prior to use.

2.7. Procedure for kinetic characteristic of CL reaction

A mixture of 0.1 ng ml^{-1} ChIFN-γ and 1.0 μg $m1^{-1}$ of HRP-labeled anti-ChIFN-γ (45 μl for each) was injected into the flow device and incubated for 20 min under stop flow. After washing the flow device with PBST for 2 min at a flow rate of 1.0 ml min^{-1} and injecting CL into flow device, the kinetic behavior of the CL reaction catalyzed by HRP labeled sandwich immunocomplex of ChIFN-γ was examined with a static method.

2.8. Immunoassay protocol

The CL immunoassay process for ChIFN-γ was illustrated Scheme 1 and given in Table S1 (Supporting information). A mixture of ChIFN-γ sample and 1.0 μg $m1^{-1}$ of HRP-labeled anti-ChIFN-γ (45 μl for each) was firstly delivered into flow device and incubated under stop flow at RT for 20 min. PBST was then delivered into the system at an optimal flow rate of 1.0 ml min^{-1} to wash the flow device. Subsequently, CL substrate solution was introduced into the flow device. When the HRP catalyzed CL reaction was triggered for 0.5 min under stop flow, CL signal was collected. The whole procedure from sample injection to signal detection could be finished within 25 min.

3. Results and discussion

3.1. Characterization of immunosensor

Due to the excellent capability for film formation, nontoxicity, biocompatibility, mechanical strength, and good water permeability (Kang et al., 2009), chitosan solution was chosen to mix with GO and then dropped on the surface of epoxy-activated glass slide to form a stable GO-chitosan nanocomposite film. The presence of chitosan provided a more favorable microenvironment for loading of proteins. The TEM image of monolayer GO sheets showed a typical flake-like shape with slight wrinkles (Fig. 1a), which was in agreement with the previous report (Wang et al., 2012). From SEM image of the GO-chitosan composite (Fig. 1b), the typical crumpled and wrinkled sheet structure could also be clearly observed in the composite film. After the loading of ChIFN-γ antibody on the film, the film showed obviously different surface morphology from GO-chitosan film with bright spots (Fig. 1c), indicating ChIFN-γ antibody was successfully immobilized.

The biocompatibility of a biosensing interface for loading and preserving the bioactivity of biomolecules is positively related to its hydrophilicity, which can be characterized by measuring the contact angle of the interface (Zhu et al., 2002). The contact angles of the piranha-treated, GPTMS grafted, GO-chitosan modified and antibody immobilized glass substrates were $37 \pm 1.2°$, $64 \pm 3.1°$, $46 \pm 1.5°$, and $28 \pm 0.6°$ with five measurements, respectively (Fig. S1, Supporting information). The silylanized glass substrate produced some epoxy groups, leading to bad hydrophilicity. The lower contact angle of GO-chitosan film than silylanized substrate demonstrated its excellent hydrophilicity, which provided a favorable microenvironment for proteins immobilization. The antibody

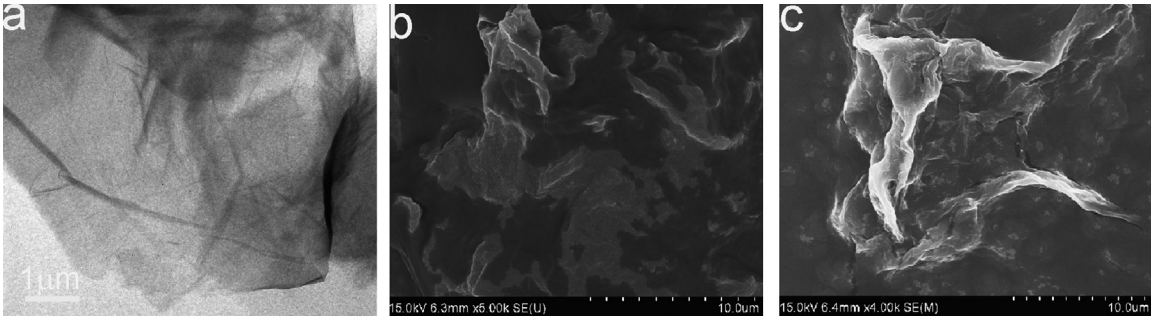


Fig. 1. TEM image of GO (a), and SEM images of GO-chitosan film (b) and anti-ChIFN-γ antibody immobilized GO-chitosan film (c).

immobilized nanocomposite displayed the smallest contact angle, indicating the successful loading of monoclonal antibody in the film. The excellent biocompatibility of the GO-chitosan film was believed to be highly advantageous to the preparation of bioreactors and biosensors (Wang et al., 2012).

3.2. Kinetic characteristic of CL reaction

HRP and alkaline phosphatase (ALP), as two major labels, are usually used in enzyme-catalyzed CL immunoassay. HRP-catalyzed CL reaction is much faster than ALP-catalyzed reaction (Fu et al., 2006), thus HRP was used in this work as an enzyme label to design CL immunoassay system for the detection of ChIFN-γ. The CL reaction immediately occurred after injecting the CL substrate into the flow device. The CL intensity quickly increased and reached its maximum value at 0.5 min (Fig. 2a). Due to the inhibition of HRP bioactivity by H_2O_2 after a long time exposure, the CL intensity slowly decreased. To obtain highest sensitivity, the enzyme-catalyzed CL emission was collected for 10 s after 0.5 min.

3.3. Incubation time

The incubation time is usually controlled by mass transport of immunoreagents and kinetics of immunoreaction, which are the key factors to improve the whole immunoassay assay speed. Compared to the microwell used in conventional ELISA, the large surface area of the GO-based immunoreactor enhanced the reaction area for formation of sandwich immunocomplex, and the thin flow cell with 0.09-cm thickness was beneficial to reducing the diffusion distance of the immunoreagents (Fu et al., 2006; Yang et al., 2008). So the immunoreagents were easy to diffuse to the surface of the immunoreactor to accelerate the rate of immunoreaction. With the increasing incubation time, the CL signal for 0.1 ng ml^{-1} ChIFN-γ increased and trended to the maximum value at 30 min (Fig. 2b), indicating the maximum formation of the sandwich immunocomplexes. This incubation process needed much shorter time than that of 1–3 h at 37 °C for the multiwell plate based ELISA (Yang et al., 2008). The CL intensity for ChIFN-γ at the incubation time of 20 min was 65% of the maximum value. To obtain the optimal analytical performance and further develop a high sample throughput method for detection of ChIFN-γ, 20 min of incubation time was used in the immunoassay to improve assay speed.

3.4. Optimization of HRP-labeled ChIFN-γ antibody concentration

The appropriate concentration of HRP conjugate is important in deciding the detection sensitivity and dynamic range of CL enzyme immunoassay. Excess HRP in the incubation solution results in overall increase in CL background and an unnecessary waste of the immunoreagents. Meanwhile, the high CL background thereby leads to a decrease in detection sensitivity. To obtain the highest

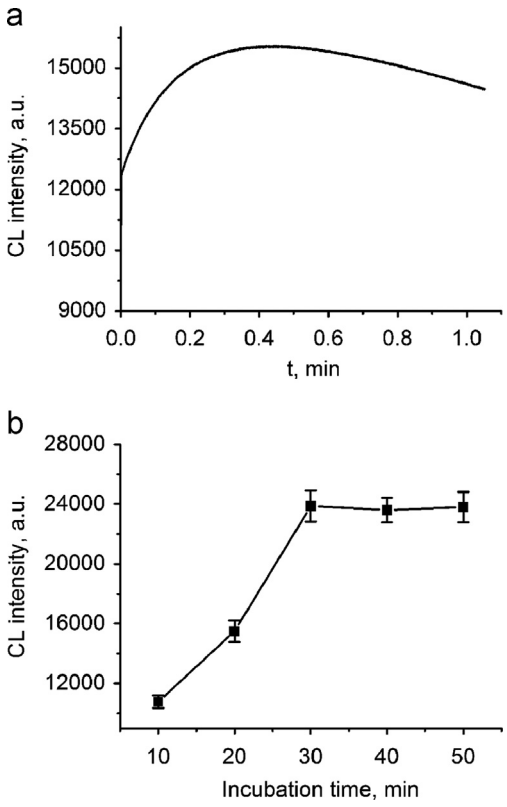


Fig. 2. (a) Kinetic curve of CL reaction catalyzed by HRP-labeled ChIFN-γ sandwich immunocomplexes and (b) effect of incubation time on CL intensity at the concentration of 0.1 ng ml^{-1} ChIFN-γ (n=5 for each point).

sensitivity and low assay cost, the concentration of the HRP-labeled ChIFN-γ antibody was optimized. As seen in Fig. 3, the CL intensity increased with the increasing concentration of HRP-labeled ChIFN-γ antibody and tended to a plateau at a concentration of 1.0 μg ml^{-1}, indicating that the available recognition sites of ChIFN-γ were matched with the enzyme conjugate. Thus 1.0 μg ml^{-1} of HRP-labeled ChIFN-γ antibody was selected as the CL immunoassay of ChIFN-γ.

3.5. Dose–response curve for ChIFN-γ

Under the optimum conditions, the CL intensity increased with increasing concentration of ChIFN-γ (Fig. 4). The dose–response curve for ChIFN-γ showed a wide linear range of 0.001–0.1 ng ml^{-1}, and the linear regression equation was I=5976.4+90675.8 [ChIFN-γ] (n=7, R^2=0.9957). The detection limit was calculated to be 0.36 pg ml^{-1} at a signal/noise ratio of 3, which was 138-fold lower than the lowest detection limit of 50 pg ml^{-1} for ChIFN-γ reported so far (Lambrecht et al., 2004). The high detection sensitivity of this method was ascribed

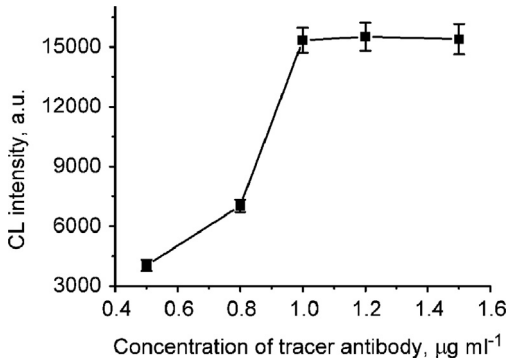


Fig. 3. Effect of HRP-labeled ChIFN-γ antibody concentration on CL intensity at 0.1 ng ml^{-1} ChIFN-γ(n=5 for each point).

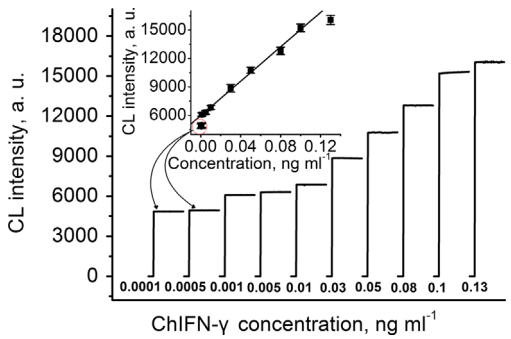


Fig. 4. Dose–response curves for ChIFN-γ. Inset: calibration curve (n=5 for each point).

to the following advantages: (i) the functionalized GO sheets increased the surface area to capture a large amount of capture antibody, thus amplifying the detection response; (ii) the excellent hydrophilicity of the GO-chitosan nanocomposite greatly reduced the non-specific adsorption. The whole immunoassay, including injecting sample, incubation, wash and detection, could be finished in 25 min, which was much shorter than the present method for ChIFN-γ (Sekellick et al., 1994; Digby and Lowenthal, 1995; Karaca et al., 1996; Lambrecht et al., 2000; Yun et al., 2000; Lambrecht et al., 2004). When coupling this method with our previous channel or support resolution strategy (Fu et al., 2008; Yang et al., 2009), the higher throughout for analytes assay could be obtained in a single assay run.

3.6. Method specificity

The specificity of the proposed method was evaluated by examining the change of CL signal at a definite concentration of ChIFN-γ upon the addition of different concentrations of analogous ChIFN-α in incubation solution. With the increasing concentration of the interferent antigen in the range of 0.05–0.2 ng ml^{-1}, the change in CL signal for 0.05 ng ml^{-1} ChIFN-γ was less than 3.5% (Fig. 5), indicating that the cross-reactivity between monoclonal ChIFN-γ antibody and the other analogous analyte could be negligible. This result showed that the constructed ChIFN-γ immunosensor had high specificity and ChIFN-γ could be selectively assayed using the designed CL immunoassay system.

3.7. Reproducibility and stability of the immunosensor

The reproducibility of the flow-through CL immunoassay system was evaluated by the intra- and inter-assay coefficients of variation (CV). The intra-assay CV was the difference among five replicative measurements of one sample using the same immunosensor, which was obtained at 0.1 ng ml^{-1} of ChIFN-γ to be 2.2%, showing good

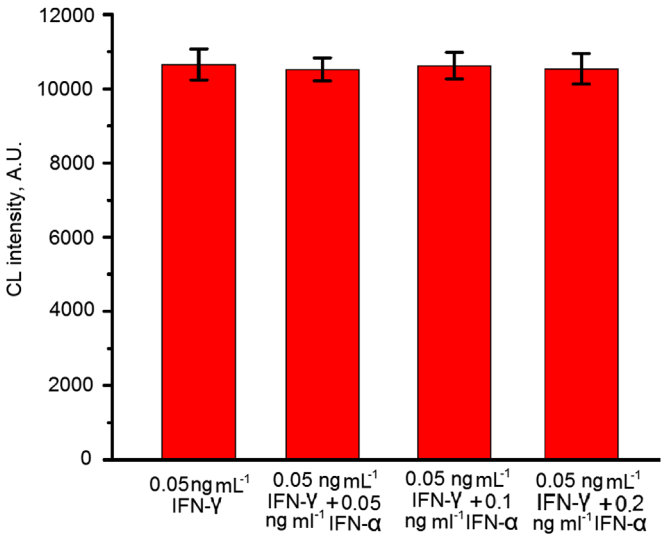


Fig. 5. CL signal of flow-through CL immunoassay system at different concentrations of IFN-α in the presence of 0.05 ng ml^{-1} of ChIFN-γ (n=5 for each point).

detection reproducibility. The inter-assay CV was the difference among the measurements of the same sample using five immunosensors prepared in batch, which was 3.4% at 0.1 ng ml^{-1} ChIFN-γ, indicating the good fabrication reproducibility.

The stability of the immunosensor was investigated over a storage period of 50 days in PBS at 4 °C. After this immunosensor was stored for 50 days, it was used to measure the CL intensity of 0.1 ng ml^{-1} ChIFN-γ. In comparison with the initial CL intensity, no obvious CL signal change was observed. This result showed that the biocompatible GO nanosheet was advantageous to retain the bioactivity of immobilized antibody in the film, which was significant for the development of the proposed method in low-cost application.

3.8. Detection of natural ChIFN-γ and infected serum samples

To evaluate the accuracy and application potential of the flow-through CL immunoassay system, the assay of ChIFN-γ in supernatant and infected serum samples were performed using the proposed method. Prior to the assay, ChIFN-γ supernatants were appropriately diluted with 0.01 M pH 7.4 PBS. The ChIFN-γ concentrations in supernatants detected with this method were 0.29 ± 0.02 and 0.82 ± 0.036 ng ml^{-1}, which shows an acceptable agreement with the values of 0.30 and 0.78 ng ml^{-1} from the reference ELISA method. The latter was performed by School of Medicine of Yangzhou University. Moreover, the recovery tested by spiking 0.01, 0.03 and 0.05 ng ml^{-1} ChIFN-γ in chicken serum samples ranged from 98.9% to 100% (Table S2, Supporting information). These results suggested that the proposed CL immunoassay system could satisfy the need for determining ChIFN-γ in complex biological fluid.

4. Conclusions

This work developed a new CL immunoassay method coupled with flow-through system for quantitative determination of ChIFN-γ. The GO-chitosan film provided a large surface area for high-capacity loading of proteins and displayed a biocompatible microenvironment for long activity retention of the biomolecules. Different from traditional biological assay and ELISA, this method displayed excellent performances such as ultrahigh sensitivity, rapid assay speed, simple manipulation and wide linear range. Up to now, the obtained detection limit (0.36 pg ml^{-1}) was the

lowest among the reported methods for detection of ChIFN-γ. Using analogous ChIFN-α as the interference analyte, the immobilized monoclonal antibody showed highly specificity for ChIFN-γ. The immunosensor also had good detection and fabrication reproducibility and acceptable stability. It could be successfully applied in the detection of ChIFN-γ in practical samples. This method supplied a promising practicality in rapid detection of ChIFN-γ for good evaluation of CMI in poultry. Significantly, the proposed method could be readily extended to the detection of other cytokines or antigens.

Acknowledgments

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Appendix A. Supporting information

Supporting information associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.07.067.

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